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REVIEW

The Genetic Analysis of Neuropeptide Signaling Systems

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INTRODUCTION

One of the important achievements of neuroscience in the last decade is the recognition that neurons release and respond to a very large number of signaling molecules. Many of these regulatory molecules are neuropeptides. They play both general and specific roles in the neural control of physiology, behavior and development. Neuropeptides are typically small molecules that are made as part of larger precursor forms; many different neuropeptides are often derived from the same precursor [101]. Such biologically active peptides are released along with classical transmitters and individual neurons are known to express and to release, in coordinate fashion, the products of several neuropeptide genes.

These features of co-synthesis and co-release of several neuropeptides suggest that they may have shared functions. Physiology and pharmacology have already revealed much about the functions of individual neuropeptides. However, we remain ignorant about the functions of the majority of neuropeptides, and are almost completely ignorant about the physiological processes that are controlled by sets of co-synthesized peptides. This feature presents an especially difficult problem for functional analysis because the biologically active agents may be working individually or in concert. Traditional analyses of neuropeptide functions are pharmacological and are often frustrated by the lack of specific antagonists by which to interrupt peptide action. Surgical experiments may remove the source of peptide secreting neurons, but such experiments must be controlled for variables unrelated to peptide action. In this regard, genetics can be extremely useful because it addresses questions regarding the function of molecules *in vivo* and should prove a useful complement to other forms of experimental analysis for the study of neuropeptide systems.

The significance of a genetic analysis stems from many features. First it is performed *in vivo*, and hence does not rely on choice of assay, nor does it preclude the analysis of multiple target sites. Second, it may address gene function in its entirety and so may consider the coordinate roles of distinct neuropeptides that are co-synthesized and released. Third, it provides the means to study animals that are chronically deficient in neuropeptide signaling, and so, can test the consequences of the disruption of such regulatory pathways. Finally, available methods permit the re-introduction of gene sequences (by germ line transformation) so as to test and extend hypotheses derived from observations of mutant phenotypes. Our intention in this review is to consider the prospects for a genetic analysis of neuropeptide signaling in simple systems, especially in the fruit fly, *Drosophila* and in the nematode, *C. elegans*. We principally focus on one gene, that encoding several FMRFamide-related neuropeptides, as a basis by which to evaluate the prospects for the approach. We survey the recent progress in the characterization and genetic analysis of other neuropeptide genes in systems amenable to genetics. Finally, we consider 'model system' studies of molecules that perform related functions within the broad category of neuropeptide signaling, and we discuss novel techniques by which genes encoding such molecules may be identified and targeted for genetic analysis.

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INVERTEBRATE *FMRFamide* GENES

FMRFamide-related peptides have been isolated from species as diverse as coelenterates and mammals. The authentic tetrapeptide, Phe-Met-Arg-Phe-amide, has been found in mollusks and annelids; most other examples are extended at the amino-terminus. In invertebrates, genes encoding FMRFamide-like peptides have been cloned in the mollusks, *Aplysia* [88, 105], *Helix* [56], and *Lymnaea* [54, 86], in the nematode, *Caenorhabditis* [84], and the fruit fly, *Drosophila* [63, 94, 95, 103]. Sequence homologies indicate they are ancestrally related to the vertebrate opioid and corticotrophin releasing factor neuropeptide genes [105]. Pharmacological studies have shown that FMRFamide and related peptides in invertebrates have diverse physiological effects: they are cardioactive [51, 75, 79] and increase neurally-induced tension in skeletal muscles (e.g., [16]). In the central nervous system, FMRFamide-related peptides alter specific motor patterns and affect behavioral states. In mollusks, FMRFamide decreases or abolishes patterned motorneuron activity involved in feeding [55, 59].

The analysis of genes encoding FMRFamide

neuropeptides in animals as different as snails, worms and insects provides an opportunity to define the differences in gene structure and expression that have evolved over considerable evolutionary time. It has been argued that such evolutionary comparisons provide a basis with which to identify functionally important regions of a gene (e.g., [4, 43]). Among these diverse species, the *FMRFamide* gene appears to have retained several salient features, but also displays a remarkable amount of species-specific gene structure.

At least two genes encode FMRFamide-related neuropeptides in *Drosophila*. *Drosophila FMRFamide* generates several extended FMRFamide sequences [12, 95]. The existence of a second gene (called *dromyosuppressin-dms*) has been inferred from peptide sequence analysis [66]. As described in a later section, *dms* is likely to encode an extended FLRFamide. The *Drosophila FMRFamide* gene has a simple structure: a single intron separates a short 5' untranslated leader from the exon encoding a single prohormone precursor [12, 95]. The entire transcription unit in flies comprises only ~6 kb and transcription appears limited to the production of a single RNA. In the nematode, *C. elegans*, Li and colleagues have defined a single copy gene (called *flp-1*) that is split into 6 exons and that encodes a small precursor from which several N-terminally extended FMRFamide-like peptides are produced [84, 92]. In the snail, *Lymnaea*, the *FMRFamide* gene spans more than 20 kb and is composed of several exons that are differentially spliced to produce alternative RNAs. In a striking finding, Burke and colleagues found that the FMRFamide RNAs in the snail alternatively produce two different polyproteins: these prohormones produce short and long forms of FMRFamide-related peptides, respectively [11, 87]. Short forms (also referred to generically as "tetrapeptides") include authentic FMRFamide and FLRFamides. Long forms (also referred to generically as "heptapeptides") include peptides 6–10 amino acids long and represent N-terminally extended forms of the FMRFamide peptide family (e.g. GDPFLRFamides, [11, 87], also see [13]). In Figure 1, we schematize the organization of these diverse *FMRFamide* genes in various species to highlight what we emphasize as differences.

The contribution of RNA splicing to the generation of FMRFamide neuropeptide diversity varies greatly according to the animal examined. As mentioned earlier, the *Drosophila* gene does not appear to be spliced into alternative RNAs. The nematode *flp-1* gene displays some alternative splicing to generate either of two precursors that differ by the presence of one additional FMRFamide-like peptide [84]. RNA splicing appears most important in the case of the molluscan FMRFamide systems: the short versus long peptide precursor phenotype of *Lymnaea* is solely determined by this mechanism.

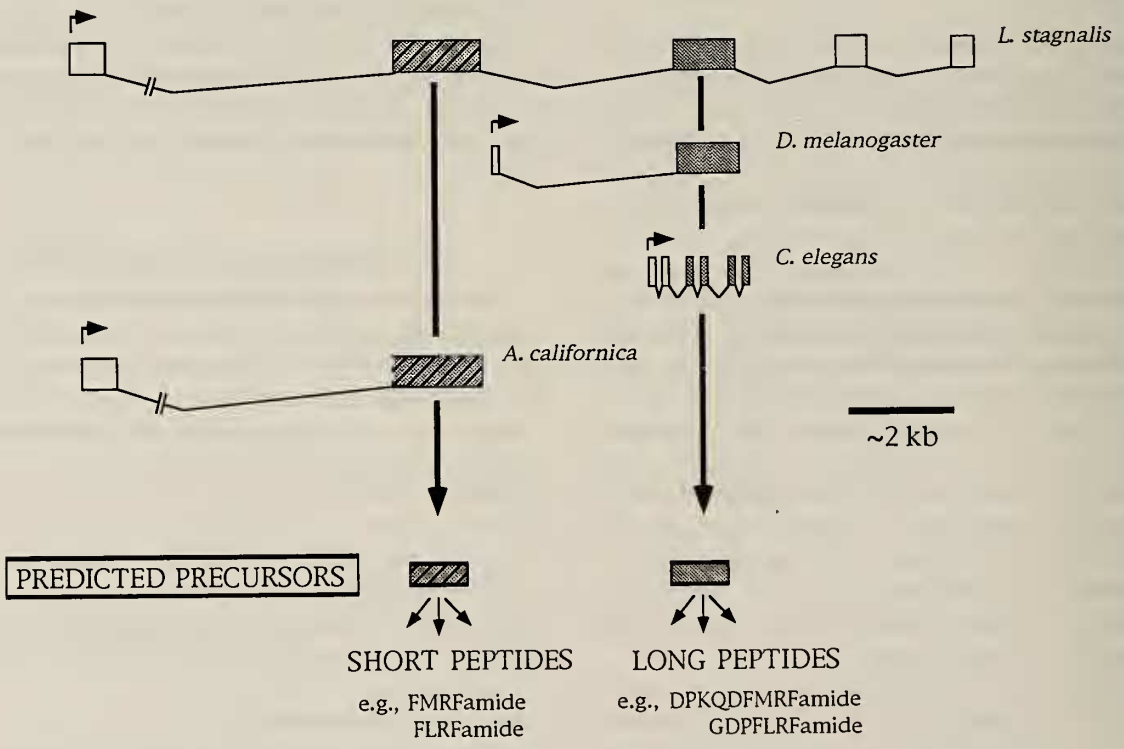

FIG. 1. Schematic organization of several genes that encode FMRFamide-related neuropeptides. Boxes indicate the position and number of exons; thin lines that connect the boxes represent intronic sequences. The sizes are only approximate representations. The small arrows at the front of genes indicate the positions of transcription start sites. In the case of *Aplysia*, the number of exons and the position of the start site are inferred (see [105]). The marking within certain exons indicates the positions of sequences that encode pro-FMRFamide precursors: the cross-hatched stippling represents exons that encode "short peptide" precursors; the stippling without cross-hatching represents exons that encode "long peptide" precursors.

The *Drosophila* and *C. elegans* FMRFamide precursors appear similar to the long (heptapeptide) precursor of the snail according to several criteria (Fig. 2). First, the length of the encoded peptides are similar and all are extended in the N-terminal direction. Secondly, the peptides are arranged similarly within the precursor, such that most are separated from each other by only a single basic residue. This residue is likely to be the site of endoproteolytic cleavage, an event which liberates the individual peptides from the precursor. In contrast, the *Lymnaea* tetrapeptide (short) precursor retains a characteristic acidic spacer region of 6 to 8 amino acids between the repeated FMRFamide coding units. In addition, the coding units themselves are most often flanked by dibasic residues. This is similar to the arrangement of the *Aplysia FMRFamide* gene which encodes multiple copies of the tetrapeptide with acidic spacer regions between each copy. Interestingly, the long (heptapeptide) forms of FMRFamide-related peptides have not been detected in *Aplysia*; whether the exon that encodes the long forms is absent in that animal (see Fig. 1) or whether this reflects a post-transcriptional change has not been defined. In the case of the snails, the fly and the worm, experiments suggest that the *FMRFamide* genes here defined are each present in single copy. It appears unlikely therefore, that flies and

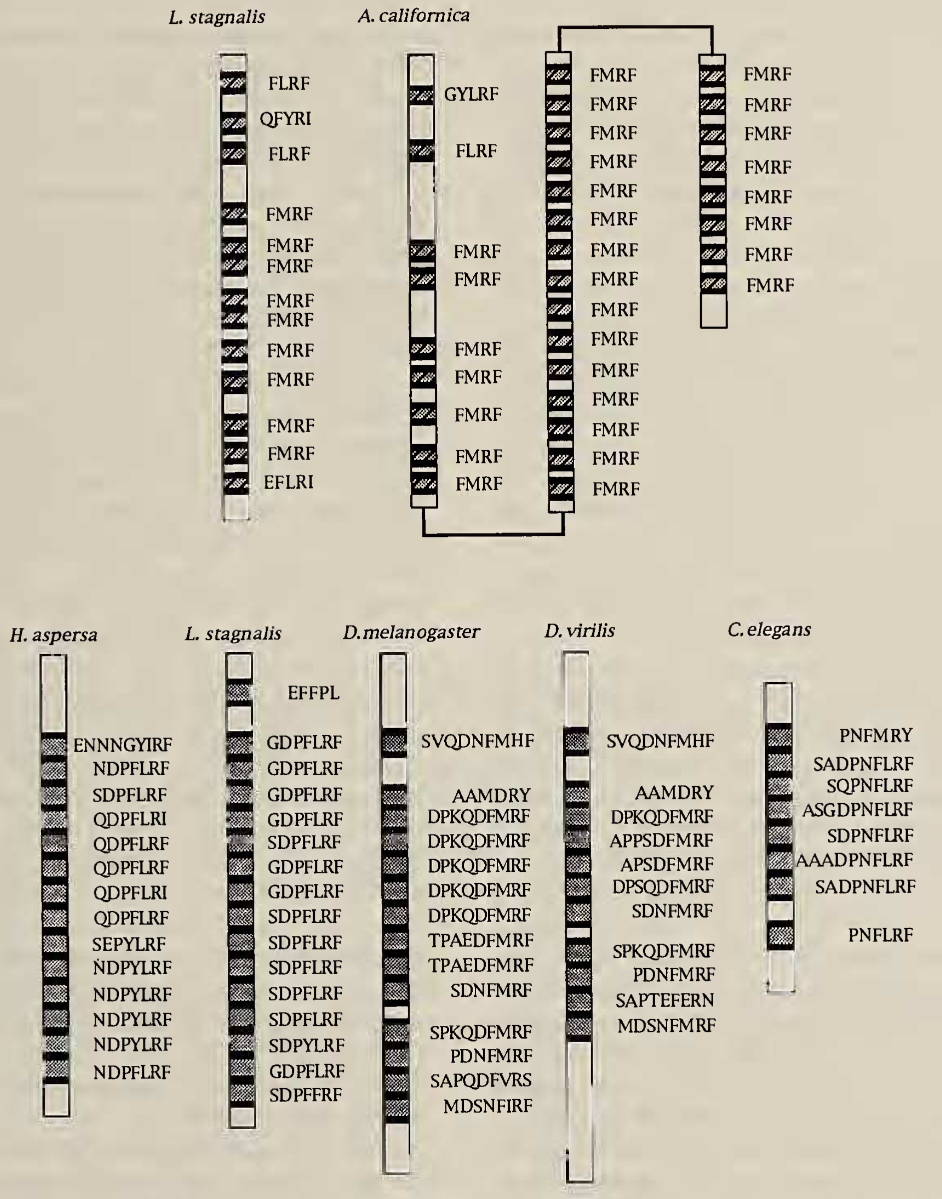

FIG. 2. Schematic representation of several precursors encoding FMRFamide-related neuropeptides. The top half of the figure illustrates the short (tetrapeptide) precursors from *Lymnaea* and *Aplysia*. The lower half illustrates the long (heptapeptides, etc.) precursors from two molluscan species, two fly species, and from the nematode. The size of the precursors are only approximate representations. The short FMRFamide peptides are denoted with cross-hatched stipples, the long FMRFamide peptides with stipples only. Basic cleavage sites are shown with black bars; amidation sites are present following each peptide (not shown). Note that the short peptides are typically separated from each other by spacer regions and the long peptides are not.

worms contain other, as yet uncharacterized, genes that are more related to the snail *FMRFamide*. Rather, this appears to be a case wherein the difference in the biology of the animals have promoted and/or permitted a divergence of *FMRFamide* gene structure.

TRANSCRIPTIONAL REGULATION OF THE *FMRFamide* GENE

The FMRFamide neuropeptide phenotype is associated with a stereotyped though heterogeneous group of neurons. This association appears to be stable over considerable evolutionary time: in flies, the pattern of neurons is highly reproducible between species separated by an estimated 60 Myr [103]. The same observation was made for distinct nematode species [93]. This strong linkage of neuropeptide phenotype with neuronal cell type suggests that the functions of the transmitters are intimately tied to the functions of particular neurons and to the synaptic circuits in which those neurons participate. We do not known whether these FMRFamide-expressing neurons participate in a common functional circuit, or whether they subserve independent roles in the animal. Nevertheless, our understanding of the rules that produce precise patterns of neuropeptide gene expression throughout development could contribute directly to the elucidation of neuropeptide function. The establishment of these individual neuronal properties requires the restriction of gene expression to specific neurons and it exemplifies a fundamental problem in developmental neurobiology. The *Drosophila FMRFamide* neuropeptide gene is a useful model with which to address this issue because its expression is restricted to a small group of neurons in the central nervous system (CNS) that is stereotyped such that individual neurons can be followed throughout development.

In vivo analyses of promoters for several neuronal genes have begun to define the mechanisms underlying such phenotypic complexity. Highly restricted patterns of reporter gene expression have been seen using regulatory fragments of neural-specific genes. For example, Young, *et al.* [110] reported that *oxytocin* gene expression was closely mimicked in mice bearing a 5.2 kb DNA fragment from the *oxytocin/vasopressin* locus. The organization of regulatory elements that underlie more widespread patterns of gene expression have also been described. Deletion analysis of the *Drosophila choline acetyltransferase* promoter revealed the presence of multiple regulatory elements, whose removal led to the absence of reporter gene expression in subsets of the normal pattern [49]. For the *Dopa decarboxylase* gene of *Drosphila*, deletion and mutation analyses have suggested that its expression by any single cell type depends on cell type-specific enhancer elements and a common, tissue-specific regulatory element that is required for expression by all *Ddc* neurons [10, 36, 43]. Similarly, in the *fushi tarazu, even-skipped*, and *sevenless* genes of *Drosophila*, numerous *cis*-regulatory elements act in concert to produce complex patterns of expression [35, 24, 5, 6].

In the CNS of the *Drosophila* larva, the FMRFamide pattern consists of ~50 neurons that are distributed throughout the brain and ventral nerve cord [97]. Following metamorphosis, there is an increase in the number of neurons expressing the gene to a total of ~120 [70, 97]. Based on position and axonal projections, there neurons represent ~15 discrete cell types including large neuroendocrine cells and interneurons in the ventral ganglion, central brain and sensory neuropils. Recent studies using reporter gene expression and germ line transformation methods [96], (M. Roberts, S. Renn and P. Taghert, unpublished) have generated evidence for three principal conclusions regarding the regulation of the *FMRFamide* gene. First, that an 8 kb fragment containing upstream and intragenic regions of the *FMRFamide* gene contains sufficient regulatory information to direct the appropriate pattern of transcription (and therefore peptide expression) to the normal complement of ~15 neuronal cell types. Second, that separate regions of this fragment are required for expression by different neuronal cell types. Third, that neuropeptide gene expression in individual cell types, the OL visual system neurons and the Tv neuroendocrine neurons, is produced by small, non-overlapping DNA regions that display the properties of cell type-specific enhancers. This information is schematized in Figure 3.

The first conclusion stems from studying the activity of an 8 kb fragment of the *FMRFamide* gene. This activity is sufficient to direct *lacZ* expression *in vivo* with a pattern and intensity that is nearly indistinguishable from that of the endogenous gene. This correspondence suggested that the pattern and level of *FMRFamide* gene expression is largely determined by transcriptional control mechanisms. Furthermore, the loss of β-gal immunoreactivity from specific cell types following transformation with the deletion constructs suggested that control regions within the *FMRFamide* promoter are distributed throughout the 8 kb of flanking and intragenic sequences (Fig. 3). The distributed organization of required elements is consistent with previous studies of tissue-specific gene expression, which have suggested that transcription is regulated by multiple modular enhancers [104, 23, 27, 25, 35, 21]. Similar to many of these studies, the *FMRFamide* gene contains a broadly distributed set of regulatory regions that appear to control different subsets of the cellular pattern. One of these regions, from −476 to −162, can act as a cell type-specific enhancer for a single neuronal type, the OL2 group [96]. Thus, within a large regulatory domain that controls gene expression by many neuronal cell types, this small region is sufficient for expression by a single cell type.

More recent studies have indicated the presence of a second cell type-specific promoter. An adjacent region of the promoter (from −922 to −476 bp) can direct reporter gene expression to a different set of FMRFamide neurons, the neuroendocrine Tv cells (M. Roberts, S. Renn, and P. Taghert, unpublished). These data suggest that certain regulatory elements of the *FMRFamide* gene are autonomous

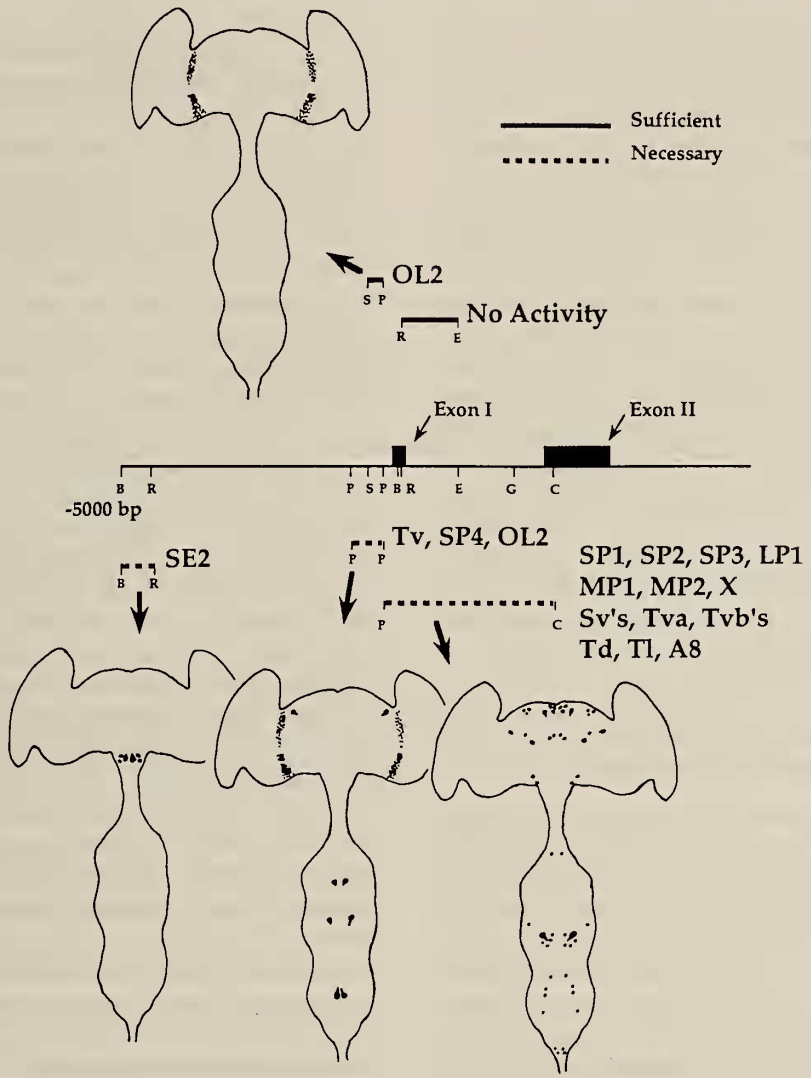

FIG. 3. A schematic that illustrates the positions of DNA regions within the *FMRFamide* promoter that control cell type-specific *lacZ* gene expression. The *FMRFamide* gene is diagrammed in the center of the figure with the two exons indicated as black boxes. Restrictions abbreviations include: B (*Bam*HI), R (*Eco*RI), P (*Pst*I), S (*Sal*I), E (*Spe*I) G(*Bgl*II), C (*Cla*I). Below the gene, the dotted lines and corresponding drawings summarize results of deletion analysis. These regions are necessary for *lacZ* expression in the indicated neurons. Above the gene, the results of the enhancer constructs are summarized. The 300 bp fragment lying 162 bp 5' to exon I is sufficient to direct expression of the reporter gene by a heterologous promoter in the OL2 neurons. The names of neuronal cell types that express the *FMRFamide* gene are presented as abbreviations (e.g, SE2, OL2, etc.); see ref. [96] for a more complete description. Reprinted with permission of Cell Press.

enhancers, capable of directing expression to individual cell types. Accordingly, not all of the diverse neurons that share this specific neuropeptide phenotype share a common transcriptional control. Instead, the parallel regulation of these distinct enhancers in different cell types results in the full pattern of *FMRFamide* expression. Some of the elements appear specific for particular cell types, while others appear to contribute to many cell types in coordinate fashion. In addition, ectopic reporter gene expression, both within the CNS and in other tissues [96], (M. Roberts, S. Renn, and P. Taghert, unpublished) indicates the presence of negative transcriptional elements as well. This model shares may features with ones that describe the activation of the pair-rule genes *hairy* and *eve* in individual stripes across the *Drosophila* blastoderm [28, 40, 74]. Certain individual stripes of gene expression are controlled by discrete enhancer regions; other stripes are coordinately controlled by a common regulatory domain.

Aspects of this model have many important implications for the regulation of *FMRFamide* gene expression. For example, the use of independent enhancers could provide a very high degree of flexibility in the evolutionary modification of gene expression, at the level of single cell types. A possible example of such evolutionary modification is illustrated by two neurons called Tv and Tva. Although these two cells share a common cell body position, axonal target,

and neuroendocrine function, different regions of the *FMRFamide* promoter are required for their respective expression (Fig. 3). Furthermore, both the Tv and Tva cells are present and morphologically differentiated in larval stages (D. Zitnan and P. Taghert, unpublished), but only the Tv neurons have detectable levels of *FMRFamide* expression at this time. In contrast, both the Tv and Tva neurons in *D. virilis* are FMRFamide-immunoreactive in larval stages [103]. This subtle difference between closely related species may result from the modified use of independent, cell type-specific enhancers. Further analysis of the regulatory domains and their molecular controls should contribute to our understanding of neurotransmitter phenotypes in the brain. As outlined below, related questions of interest include those of comparative regulation: How similar are the regulatory domains for different neuropeptide genes? When coordinately expressed in the same neuronal cell type, are they independently or jointly regulated? The precision of studies of identifiable neurons of simple systems, when combined with the power of genetic transformation techniques, makes it now possible to address such fundamental questions directly.

GENETIC ANALYSIS OF NEUROPEPTIDE FUNCTIONS

As described earlier, neuropeptide systems are not easily dissected because they display complex patterns of cellular expression and because they employ several strategies to generate diversity of peptide sequences. For this reason, genetics could be useful to analyze neuropeptide systems at many levels. There are thirteen copies of structurally-related neuropeptides on the *Drosophila* proFMRFamide precursor. Many of the individual peptides are indeed processed from the precursor, as determined by purification from tissue homogenates [63, 66]. This arrangement of multiple, biologically active molecules is reminiscent of numerous neuropeptide precursor structures. The proenkephalin and prothyrotropin-releasing hormone precursors of mammals, for example, each contain multiple copies of their respective biologically active peptide sequences [26, 50].

A genetic analysis will allow us to ask fundamental questions about the role(s) of a complex neuropeptide precursor *in vivo*. First, does the gene serve an essential function? By studying animals that are chronically deficient in the production of these molecules, we hope to learn if the survival of the animal depends on their function, or whether other neuropeptides share overlapping functions. Also, to what extent do the different co-synthesized neuropeptides act coordinately? Does the inclusion of many related neuropeptides on one precursor indicate functional amplification, or redundancy? Further, does the gene play a similar role in all the neurons in which it is expressed, or is it contributing to the execution of diverse neural activities? By manipulating gene structure in a mutant background, it should be possible to assess the importance of the sequence, copy number and arrangement of specific neuropeptides on a complex precursor molecule.

While very little information is available concerning the roles of insect FMRFamide peptides, some possible functions have been suggested by direct pharmacological analysis. In *Drosophila*, several of the neuropeptides encoded by the proFMRFamide precursor modulate neurally-evoked tension in larval somatic muscle (R. Hewes and P. Taghert, unpublished). In locusts, FMRFamide and related peptides have similar effects on skeletal muscle contractions in the leg [19]. In the blowfly, *Calliphora*, some but not all endogenous FMRFamide-like neuropeptides induce fluid secretion from isolated salivary glands [18]. Whether these diverse *in vitro* tissue reponses correspond to actual physiological events *in vivo* awaits genetic confirmation.

Genetic studies of *Drosophila FMRFamide* have begun to define its chromosomal locus (Fig. 4). The gene is present in single copy and is located at position 46C of the 2nd chromosome [94]. No mutant stocks were available, so deficiency stocks were first generated which have relatively large deletions in the surrounding region. These served to define the locus and to provide a starting point for mutagenesis experiments. Ultimately, these deficiency stocks helped to define a 50-60 kb region surrounding the *FMRFamide* gene and they were used to identify lethal mutations residing within this interval [71]. Because so little is known about the functions of *Drosophila FMRFamide*, we could not accurately predict the severity or quality of phenotypes from a hypomorphic or null allele. We mutagenized flies with ethyl methane sulfonate which generally produces point mutations. Screening for the most severe phenotype (lethality) was the first step and resulted in the identification of three genes within the 50–60 kb surrounding region which give a lethal phenotype when mutated (Fig. 4). These three genes with lethal mutant phenotypes appear to be neighbors of *FMRFamide*, but not *FMRFamide* itself. These results raised the possibility that the *FMRFamide* gene may not be an essential gene; they also point out two principal limitations to the identification of neuropeptide gene mutations.

The first limitation involves the probability of mutating a complex neuropeptide gene to a null phenotype by chemical mutagenesis. Such genes encode polyprotein precursors that, by their design, incorporate redundant sequence elements (e.g., Fig. 2). This design feature reduces the chances that a single base pair alteration will necessarily affect the basic functioning of the entire protein. This feature may also operate in wild type populations to lessen the consequences of spontaneous mutations due to environmental mutagens. The second complication involves the likelihood that a profound phenotype will result from a *bona fide* mutation in a neuropeptide gene. While the logic of neuropeptide signaling is yet to be defined, it almost certainly includes highly redundant signals that may be cosynthesized and/or derived from distinct precursors. This functional redundancy reduces the chances that the absence of any given group of neuropeptides will generate a distinctive phenotype.

Given these limitations, it is useful to consider alterna-

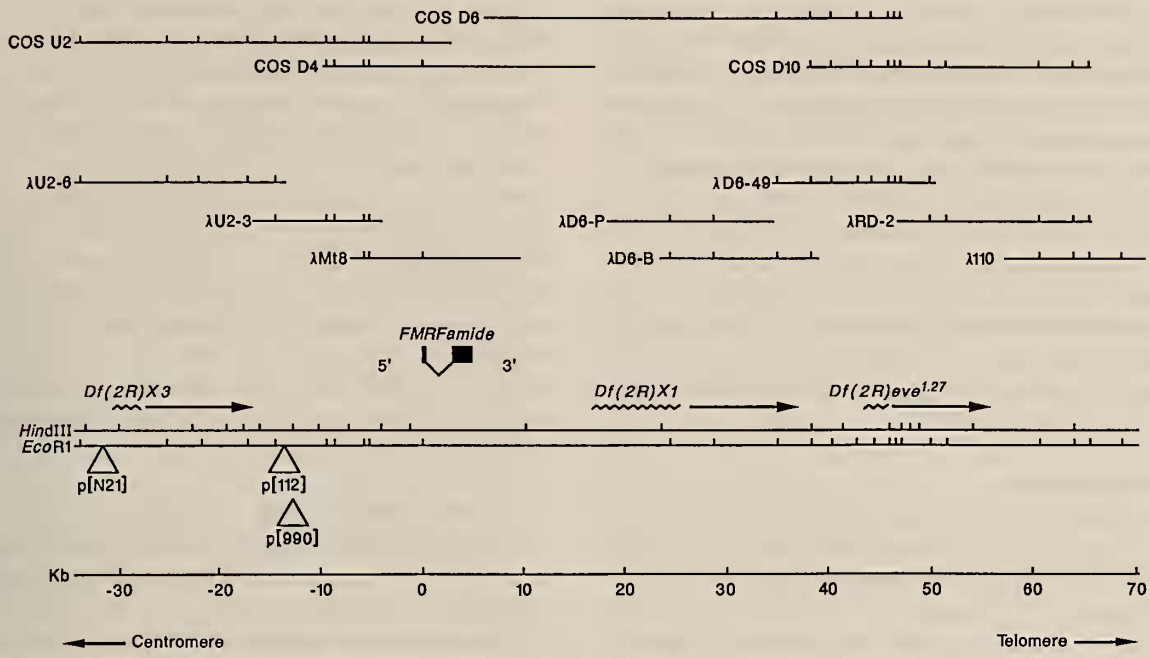

FIG. 4. Genomic map of the 46C region surrounding the *FMRFamide gene*. Cosmid (Cos) clones and lambda (λ) phage clones are shown above the schematized *FMRFamide* gene. *Hind* III and *Eco*RI restriction maps are illustrated below the schematized gene. Deficiency endpoints are denoted by a wavy line and the direction of the deletion from the endpoint is noted by an arrow. P element insertions are illustrated with a triangle and the insertion designation. (See ref. [71]). Reprinted with permission of the Genetics Society of America.

tive methods to analyze neuropeptide systems genetically. In this regard, transposable elements offer useful complimentary approaches to initiate novel genetic analyses. Insertional mutagenesis is useful because it can be employed as a molecular screen and therefore is not dependent on the ability to predict a phenotype. For example, with respect to the *Drosophila FMRFamide* gene, a "local hop" strategy of mobilizing P elements [107] was used to identify a stock bearing an insertion ~15 kb upstream of the gene [71]. This insertion disrupts a neighboring gene and hence is not useful by itself to define FMRFamide functions. Nevertheless, such proximity encourages the hope that reiterations of the experiment will produce the more specific insertional event desired. A similarly-conceived screen in *C. elegans* has resulted in the identification of a stock bearing a transposable element (Tc1) inserted within the *flp-1* promoter (C. Li, per. communication). Excision of this insert should permit a detailed examination in the near future of worms that are deficient in the production of FMRFamide neuropeptides. The utility of transposable elements for the analysis of neuropeptide systems is also evident in the use of enhancer trap screens, as we discuss in a later section.

OTHER NEUROPEPTIDE SYSTEMS IN *DROSOPHILA*

We have outlined studies of the *FMRFamide* gene in model systems as a means of introducing outstanding questions relating to neuropeptide structure, expression and functions. Molecular genetic studies have also been initiated for several other *Dorosophila* neuropeptide genes, including those encoding the drosulfakinins [65], the eclosion hormone [39], and the adipokinetic hormone [90]. Their chromosomal positions are 81F, 90B, and 64AB, respectively. A gene encoding dromyosuppressin, an extended FLRFamide-related neuropeptide, has also been inferred [66], and is discussed due to its potential relationship to the *FMRFamide* gene. Genetic analyses in all these cases are in their initial stages; however, the available molecular information provides the basis for addressing questions about function and regulation of expression. Notably, this section does not review several pertinent studies of neuropeptides in *Drosophila* for which the genes have not yet been cloned. We refer the reader to a recent review by Nässel [64] for a discussion of this substantial body of information.

I. Drosulfakinin

Questions regarding function and regulation are especially pertinent for neuropeptides derived from conserved evolutionary families. Among neuropeptide systems being investigated in *Drosophila*, in addition to FMRFamide, drosulfakinin, is also a member of an -RFamide family with apparent homologues in both vertebrates and invertebrates. The *drosulfakinin* (*dsk*) gene [65] was cloned using oligonucleotide probes based on the conserved amino acid sequences of the vertebrate gastrin and cholecystokinin (CCK) peptides, as well as another insect neuropeptide, leucosulakinin, from the cockroach [60, 61]. Two of three encoded peptides in the pro-dsk precursor are *Drosophila* homologues of the gastrin and CCK family of vertebrate peptides, while

the third is novel [65]. Two features suggest that insect dsk and vertebrate gastrin/CCK systems may display conservation of functions and/or receptor structure. First, the closest homology resides in the C-terminal pentapeptide and there is a tyrosine just N-terminal to the pentapeptide. The C-terminal pentapeptide and the sulfated tyrosine are thought to be necessary for activity of the gastrin/CCK peptides [62]. Secondly, in vertebrates, gastrin and CCK are gastrointestinal-brain peptides with implicated functions in digestion, feeding behavior and satiety [100]. A dsk peptide also has been localized in the brain [106] and gut [67]. A genetic analysis will be essential for determining if the expression of the dsk peptides reflects a conserved function in digestion and feeding behavior.

II. Dromyosuppressin

Dromyosuppressin (dms) was purified from *Drosophila* extracts with an anti-RFamide antibody [66]. Its sequence (TDVDHVFLRFamide) was not among those deduced from the *FMRFamide* or *dsk* genes; this indicated a separate genomic locus. The dms peptide shares sequence homology with leucomyosuppressin from the cockroach [37], and neomyosuppressin from the flesh fly [22], peptides that both have myoinhibitory effects. A specific antibody against the TDVDHV portion of the peptide was generated and used to demonstrate that this peptide is expressed in the CNS and in the gut [57]. The degree to which the *dms* and *FMRFamide* genes are evolutionarily related will be an important issue to resolve. Dms does not co-localize with the dsk peptides [106]; the possibility of co-localization with the peptides encoded by *FMRFamide* has not yet been addressed. Co-localization of distinct RFamide-neuropeptides would support a hypothesis that these genes may have retained similar promoter/regulatory regions. Equally important are the functional consequences derived from the relationship between *FMRFamide* and *dms*. A genetic analysis of these two genes in flies will be important for determining the degree to which their functions are unique and non-overlapping.

III. Eclosion hormone

Eclosion hormone (EH) is a neuropeptide that triggers ecdysis behaviors in insects [109]. The *EH* gene in *Drosophila* was cloned by homology using sequences from the tobacco hornworm, *Manduca sexta* [39]. In both species, the precursor is remarkably simple: a signal sequence is followed by a single, conserved 62 amino acid peptide (there is a possible additional 9 amino acid cleavage product in *Drosophila* [38, 39]). The *EH* gene has an extremely discrete expression pattern in both *Manduca* and *Drosophila*; transcripts are found in only two pair of neurons in the brain of *Manduca* [38] and in one pair in *Drosophila* [39]. In *Manduca*, the EH cell axons exit the proctodeal (hindgut) nerve along which there are neurohaemal release sites. However, it has also been established that these neurons release EH directly within the CNS from their axonal processes. The role of EH in triggering ecdysis behaviors is primarily accomplished *via* this direct access to the CNS [34]. While much useful information concerning EH action has accumulated from classic pharmacological and physiological experiments, the genetic analysis of EH could address more directly its long term roles in neuromodulation (e.g., in releasing stage-specific behaviors). Also, the full scope of its actions at non-neuronal sites could be defined; several non-neuronal target tissues for the neuropeptide have already been identified [34, 77, 99]. Likewise, a molecular genetic analysis of *EH* could address the basis for its highly restricted spatial expression and its potential regulation by genes involved in generating circadian outputs [83]. Existing deficiency stocks for the *EH* chromosomal locus, and a well-defined function *in vivo* provide a substantial basis for initiating these genetic studies.

IV. Adipokinetic hormone

The *adipokinetic hormone* (*AKH*) gene encodes a neuropeptide involved in lipid mobilization during flight in insects [102]. The gene was cloned in *Drosophila* based on the known peptide sequence of *Drosophila* AKH [89, 90]. In the grasshopper and locust, there are two closely-related forms of AKH (called I and II). They are the products of different genes, although the genes display some similarities [68, 69]. AKH I and II are co-localized and co-released, but they are not synthesized in equivalent amounts and the ratios of the two forms change during development. The precise regulation of the AKH I and II peptide stoichiometry throughout development is maintained *via* several mechanisms [72].

First, dimerization of the two precursors contributes to the stoichiometry of the processed peptides. AKH I and II are synthesized as precursors including a signal peptide, the AKH peptide, and a carboxy-terminal peptide or AKH precursor related peptide (APRP) of unknown function [32]. The precursors dimerize both as homodimers and heterodimers before the processing cleavage steps, thus producing AKH I, AKH II, and three dimeric APRPs [31, 32, 98]. Secondly, there are additional transcriptional and translational mechanisms to regulate the stoichiometry [20]. Finally, there is an increase in the number of AKH expressing cells during development, which increases the overall levels [48]. Interestingly, these co-localized and co-expressed genes have quite different 5' flanking regions, suggesting they have evolved different regulatory mechanisms [69]. Despite a considerable understanding of the mechanisms underlying this precise stoichiometry of the AKHs and APRPs, the utility of this complex peptidergic system is not well defined. The *in vivo* functions of AKH II and the APRPs are not known; it is hypothesized that AKH II has a larval function and that the APRPs may be involved in other metabolic processes related to flight [72]. In *Drosophila*, there appears to be a single *AKH* gene [90]. A comparison of its promoter region with that of the grasshopper AKH I and II promoter regions could provide insight into the evolution of distinct regulatory regions of highly related genes. The AKH-

APRP arrangement within the precursor is preserved in *Drosophila* although the APRP peptide sequence is not well-conserved. A genetic dissection of the *Drosophila adipokinetic hormone* gene would help to evaluate the physiological significance of their co-synthesis.

NEUROPEPTIDE GENES IN OTHER INSECTS

The study of neuropeptides at the biochemical, physiological, and developmental level has an extensive history in insects [47, 108]. More recently these studies have taken a molecular approach, thus offering the prospect of a genetic analysis of several additional neuropeptide systems. Two neuropeptide genes, recently cloned in other insects, are important in the neuroendocrine pathways controlling molting and metamorphosis, and may have homologues in *Drosophila*. These are the *prothoracicotropic hormone* (*PTTH*) gene, cloned from the silkmoth [1, 45] and the *allatostatin* (*AST*) gene, cloned from the cockroach [17]. PTTH, initiates the molting process by stimulating synthesis of ecdysone (the molting hormone) by the prothoracic glands. AST inhibits juvenile hormone synthesis by the corpora allata. Antibodies that display specificity to these two important developmental neurohormones both cross-react in *Drosophila* [111]. Furthermore, CNS extracts from *Drosophila* stimulate ecdysone production by the ring gland. This suggests an endogenous PTTH-like activity [33, 73]. The degree to which the neuroendocrine circuits regulating molting and metamorphosis are conserved between lepidopterans and dipterans has not been resolved (see [78]). A molecular genetic approach to this system should be very informative. Two additional neuropeptide genes cloned in other insects are considered in detail here because they illustrate two prominent features of neuropeptide systems that have not been presented in our previous examples: 1) peptides co-synthesized on the same precursor serving distinct temporal functions and 2) neuropeptide genes comprising a multi-gene family.

The *diapause hormone* (*DH*) neuropeptide gene, recently cloned in *B. mori*, [46, 85] and *H. zea* [15] provides a striking example of distinct functions for peptides encoded on the same precursor. Sequence analysis of the deduced DH precursor revealed that a distinct bioactive neuropeptide, the pheromone biosynthesis activating neuropeptide (PBAN), was co-synthesized. Although PBAN and DH are structurally related (they share a pentapeptide C-terminus), at least some of their functions appear to be distinct at different developmental stages. Diapause hormone induces diapause in the embryo in *Bombyx* while PBAN stimulates pheromone biosynthesis in the adult; thus peptides derived from a common precursor may have very distinct times at which they function [85]. Whether these are the exclusive functions of these peptides, or whether they have additional overlapping functions is not known. Such questions await answers in a genetically tractable system, like *Drosophila*, wherein the composition of a neuropeptide precursor may be manipulated and then returned to the animal for evaluation *in vivo*. While the biological functions of putative DH and PBAN homologues in *Drosophila* have not been described, this issue of functional complexity applies to any of several neuropeptide precursors that generate diverse final products.

The neuropeptide genes currently being investigated in *Drosophila* display much variation in precursor complexity, and in the degree to which the peptides they encode have retained sequence conservation through evolution. It is notable however, that they are similar in that each represents a single copy within the haploid genome. For comparative purposes, it is worth mentioning that in other insects and in other animals, neuropeptide genes are often members of large gene families (e.g., [91]). Bombyxin, an insulin-related neurohormone from the silkmoth, *Bombyx mori*, provides an extreme example of a neuropeptide multigene family. Purification of the neurohomone revealed that there were several isoforms of the protein; it is now known that this heterogeneity is due in large part to genetic diversity [41]. There are numerous bombyxin genes (>20), at least four of which were found clustered in the genome [44]. These genes are categorized into four families [41, 42]; genes are paired such that distinct members of two related families are adjacent and in the opposite orientation [44]. This arrangement could facilitate coordinated expression of related gene families. It is hypothesized that different pairs of bombyxin genes are expressed at different stages of development. A neuropeptide multi-gene family has not been identified in flies, but a molecular genetic analysis of such a gene family could help us to understand its functional significance and its regulatory interactions.

RECEPTORS AND PROCESSING ENZYMES

The enzymes responsible for processing of neuropeptide precursors and the receptors with which neuropeptides interact are important control points in the functional circuits underlying neuropeptide signaling. Their involvement would also be better defined by a molecular genetic analysis. Because genes of both types of molecules have been cloned in *Drosophila*, we include a brief summary of that information here.

Two genes related to the mammalian *furin* processing enzyme genes have been cloned (D*fur1* and D*fur2*; [30, 80, 81]. The furin protein is related to the yeast protein which is a serine endoprotease, with specificity for paired basic amino acid residues. There are four transcripts of the D*fur1* gene generated by alternative splicing [82]. *In situ* hybridization to two of these transcripts indicated that they have non-overlapping expression patterns in the embryo, suggesting different physiological roles [82]. With multiple proprotein processing enzyme genes and multiple transcripts from these genes, there may be a high degree of specificity in processing of *Drosophila* proproteins, and neuropeptide precursors, in particular. An *in vivo* mutational analysis of this rapidly expanding family of peptide processing genes will be an

important step in sorting out this specificity.

The identification of diverse neuropeptide receptors is beginning to match the diversity of neuropeptides themselves in vertebrates. This field is ripe for investigation in an organism that readily allows for genetic manipulation. To date, three identified neuropeptide receptors have been cloned in *Drosophila*, two different tachykinin receptors [52, 58] and a neuropeptide Y receptor [53]. These three receptors are developmentally regulated with peaks of expression in late embryogenesis. The spatial and temporal regulation of neuropeptide receptors clearly may have functional consequences for neuropeptide action; this is another reason why *in vivo* studies of all components of neuropeptide systems are essential for a comprehensive understanding of the function of this diverse class of signaling molecules.

ENHANCER DETECTOR LINES REVEAL DISCRETE NEUROENDOCRINE NEURON EXPRESSION PATTERNS

An alternative approach to a genetic screen for genes involved in neuropeptide signaling is a molecular screen. The advantage of a molecular screen is that it does not require consideration of potential mutant phenotype. For example, P element enhancer detector lines in *Drosophila* can be screened for expression patterns that include identified peptidergic neurons. In these lines, a β-galactosidase gene construct inserts into the fly genome. The reporter gene is constructed to require enhancer activity for robust expression; in principle, that activity derives from single neighboring genes of the host [2, 3, 29]. The significance of this method derives not only from the ease with which novel genes may be cloned, but also from the provision of direct means by which they may be subsequently mutated. The P element insertion can be mobilized and imprecise, mutating excisions can be detected (e.g., [7, 71]).

In insects, a certain class of peptidergic cells are often distinguished by their size, their axonal projections, and the large amount of secretory neuropeptides that they synthesize and store [64]. We utilized these anatomical features to screen several hundred nervous systems from developing adult *Drosophila* in which a GAL4-containing P element [9] had been mobilized. From this small-scale screen, we found several interesting patterns that are dominated by the inclusion of previously identified neuroendocrine neurons (P. Taghert, A. Schaefer, and M. O'Brien, unpublished). It should be emphasized that in most cases, the pattern of expression is not exclusive to these neurons, or even to the nervous system. The search image that we employed simply required a high level of expression and some exclusivity to certain identifiable, peptidergic neurons.

In Figure 5 is shown examples of two patterns - a general and a specific. The first is numbered P9 and it displays a pattern of gene expression in one to two hundred neurons scattered throughout the CNS. These include several identified peptidergic neurons as indicated by (i) co-expression of peptide immunoreactivities: for FMRFamide [103], allatostatin [111], or proctolin (P. Taghert, unpublished); or as indicated by (ii) axonal projections that, by terminating is neurohaemal sites, indicate a neuroendocrine, peptidergic function [64, 97]. We hypothesize that the indicated gene product may be a protein that is widely expressed by cells that secrete large amounts of peptides. There are several identified peptidergic neurons that do not express this reporter

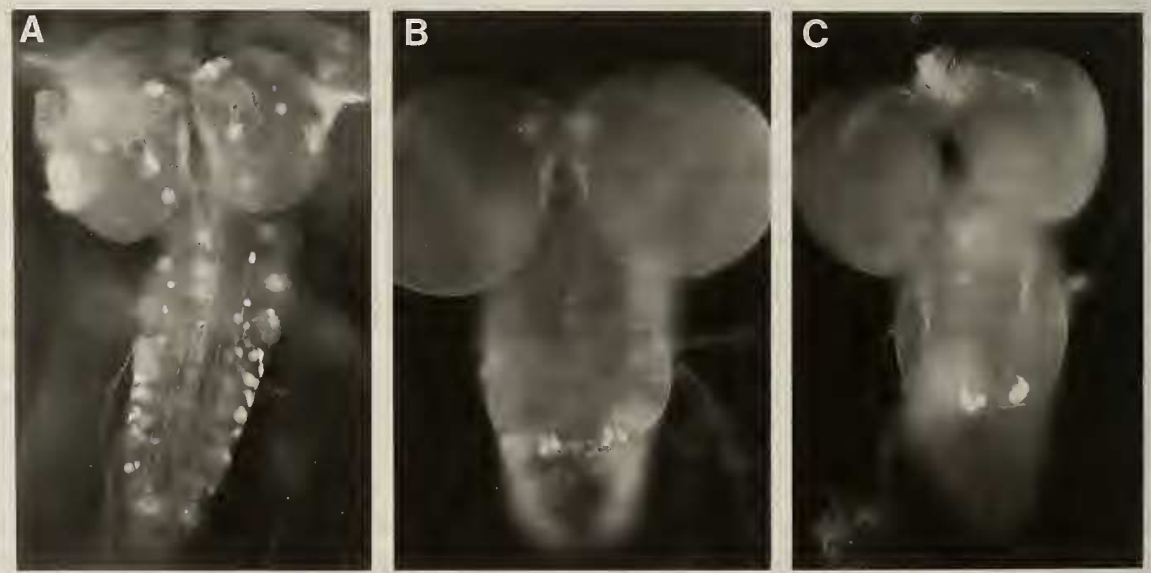

FIG. 5. Enhancer trap expression of beta-galactosidase in two lines of *Drosophila*. (A) The P[9] line is expressed in >100 neurons of the larval CNS; many are identifiable by their axonal projections and/or by their peptide immunoreactivities as peptidergic neurons. (B) The P[3] line expressed beta-galactosidase at low levels in many cells of the abdominal neuromeres of the CNS and at high levels by 14 large identifiable neuroendocrine neurons. Six of these neurons are also specifically labeled by antibodies to the molluscan neuropeptide myomodulin, as shown in (C). All examples shown are 3rd instar larval CNSs that were stained with anti-β-gal antibodies (A and B) or anti-myomodulin antibodies (C); primary antibodies were visualized by subsequent application of a fluorescent secondary antibody.

gene. Therefore, if the P9 gene serves a general secretory function, it may not be unique in its capacity.

The more specific pattern, numbered P3, displays very weak expression in widespread regions of the CNS and very strong expression in fourteen specific neurons; twelve of these are identified peptidergic neurons. In addition, there is peripheral expression in four peritracheal cells [29] per segment. An antiserum to the molluscan neuropeptide myomodulin [14] specifically labels twenty neurons in the *Drosophila* CNS, and these include six of the fourteen P3-positive neurons; this same antibody also labels two of the four P3-positive peritacheal cells per segment (M. O'Brien and P. Taghert, unpublished). In all, there is a remarkable coincidence to the spatial patterning of P3 and molluscan myomodulin-immunoreactivity. These limited observations indicate that there is a large amount of useful information to be acquired from the further examination and manipulation of enhancer detector lines.

CONCLUSIONS

In this review, we have summarized the strengths and weaknesses of analyzing neuropeptide signaling systems with genetic techniques (see also [76]). In all, we believe that the advantages far outweight the difficulties of initiating such studies. Beyond describing their sequences and their spatial patterns of expression, there are several fundamental properties of neuropeptides that remain to be defined. These include: the precise roles that such molecules play *in vivo*; the degree to which these physiological and bahavioral roles are effected by groups of neuropeptides acting in concert; whether precise neuropeptide expression (i.e., a close association with specific synaptic circuits) is a critical feature of their functioning (see [8] for a recent discussion of this point); the degree to which other molecules involved in signaling (e.g., biosynthetic enzymes, neuropeptide receptors) are points of regulation for proper signaling processes. The ability to manipulate transmitter gene expression *in vivo* will be the principal means of defining these properties and thus evaluating the contributions of neuropeptide signaling systems. Among the model systems that are amenable to genetics, there exists a wealth of interesting physiology, development, and behavior for which diverse neuropeptide systems have been indicated to play major regulatory roles. With the advent of molecular genetic techniques with which to manipulate neuropeptide signaling systems *in vivo*, we can look forward to substantial progress in further elucidating chemical signaling within the nervous system.

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